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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 39/00, 39/385, C07K 19/00

A1

(11) International Publication Number: WO 95/05849

(43) International Publication Date: 2 March 1995 (02.03.95)

(21) International Application Number: PCT/DK94/00318

(22) International Filing Date: 25 August 1994 (25.08.94)

(30) Priority Data:
0964/93 26 August 1993 (26.08.93) DK

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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

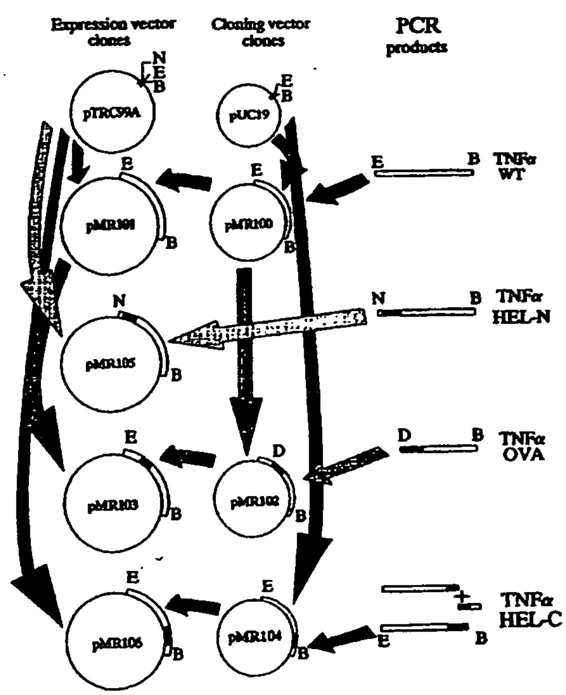
Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL EPITOPES

(57) Abstract

A novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins consists in inserting one or more foreign T-cell epitopes in such proteins by molecular biological means, thereby rendering said proteins immunogenic. The modulated self-proteins can be used as autovaccines against undesirable proteins in humans or animals, said autovaccine being useful as vaccines against a number of diseases, e.g. cancer, chronic inflammatory diseases, rheumatoid arthritis, inflammatory bowel diseases, allergic symptoms or diabetes mellitus.

Cloning strategy for murine TNFα mutants.



Restriction enzyme symbols: E: EcoRI, B:BamHI, N: NcoI, D: DraIII.

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INDUCING ANTIBODY RESPONSE AGAINST SELF-PROTEINS WITH THE AID OF FOREIGN T-CELL EPITOPES

Background of the invention

This invention concerns a novel method for utilizing the immune apparatus to remove and/or down-regulate self-proteins, the presence of which somehow is unwanted in the individual. These could be proteins which are causing disease and/or other undesirable symptoms or signs of disease. Such proteins are removed by circulating auto-antibodies which specifically are induced by vaccination. This invention describes a method for developing such autovaccines.

Introduction

Physiologically, the vertebrate immune system serves as a defence mechanism against invasion of the body by infectious objects such as microorganisms. Foreign proteins are effectively removed via the reticuloendothelial system by highly specific circulating antibodies, and viruses and bacteria are attacked by a complex battery of cellular and humoral mechanisms including antibodies, cytotoxic T lymphocytes, Natural Killer cells, complement etc. The leader of this battle is the T helper (T_H) lymphocyte which, in collaboration with the Antigen Presenting Cells (APC), regulate the immune defence via a complex network of cytokines.

Normally the individual's own proteins (the so-called self- or autoproteins) are not attacked by the immune apparatus. The described events thus generally are beneficial to the individual, but in rare cases the process goes wrong, and the immune system turns towards

the individual's own components, ventually leading to an autoimmune disease.

The presence of some self-proteins is, however, inexpedient in situations where they, in elevated levels, induce disease symptoms. High levels of immunoglobulins of the IgE class are e.g. known to be important for the induction of type I allergy, and tumor necrosis factor α (TNFα) is known to be able to cause cachexia in cancer patients and patients suffering from other chronic diseases (H.N. Langstein et al., Cancer Res. 51, 2302-2306, 1991). TNFα also plays important roles in the inflammatory process (W.P. Arend et al., Arthritis Rheum. 33, 305-315, 1990). Hormones in sex-hormone dependent cancer are other examples of proteins which are unwanted in certain situations. This invention concerns a method for the development of autovaccines against such proteins.

Others have developed autovaccines by conjugating self-proteins or appropriate synthetic peptides derived from these to large, foreign carrier proteins. Talwar et al. (G.P. Talwar et al, Int. J. Immunopharmacol. 14, 511-514, 1992) have been able to prevent reproduction in women using a vaccine consisting of a conjugate of human chorionic gonadotropin and tetanus toxoid. There are also other examples of such autoimmunogenic conjugates which have been used therapeutically in man and in animal models (D.R. Stanworth et al., Lancet 336, 1279-1281 (1990)). In the present invention the production of such conjugates between the self-proteins and foreign proteins is not necessary in order to obtain strong autoantibody responses. This has several advantages.

The technical field

T_H lymphocytes recognize protein antigens presented on the

surface of the APC. They do not recognize, however, native antigen per se. Instead, they appear to recognize a complex ligand consisting of two components, a "processed" (fragmented) protein antigen (the so-called T cell epitope) and a Major Histocompatibility Complex class II molecule (O. Werdelin et al., Imm. Rev. 106, 181 (1988)). This recognition eventually enables the T_H lymphocyte specifically to help B lymphocytes to produce specific antibodies towards the intact protein antigen (Werdelin et al., supra). A given T cell only recognizes a certain antigen-MHC combination and will not recognize the same or another antigen presented by a gene product of another MHC allele. This phenomenon is called MHC restriction.

Self-proteins are also presented by the APC, but normally such fragments are ignored or not recognized by the T helper lymphocytes. This is the reason why individuals generally do not harbour autoantibodies in their serum.

It is, however, possible artificially to induce antibodies against self-proteins. This can be done, as previously mentioned, by covalent conjugation of the self-protein to an appropriate carrier protein as e.g. tetanus toxoid or key-hole limpet hemocyanin. During the processing of such conjugates in the APC, the necessary T_H lymphocyte stimulatory epitopes are provided from the foreign protein eventually leading to the induction of antibodies against the self-protein as well as against the carrier protein. One disadvantage of using this principle is, however, that the antibody response towards the self-protein will be restricted due to shielding of epitopes by the covalently linked carrier protein. Another disadvantage is the increased risk of inducing allergic side-effects due to the contemporary induction of a very strong antibody response against the foreign carrier protein. This strong antibody response might also be the reason why this method

is not as efficient as observed in the method according to the invention.

Other researchers have coupled a single peptide T cell epitope chemically to a self-protein and managed to induce an autoantibody response with MHC restriction to that particular T cell epitope (S. Sad et al., Immunology 76, 599-603, 1992). This method seems to be more effective compared to coupling of large carrier proteins. However, it will only induce antibodies in a population expressing the appropriate MHC molecules. This means that a rather large number of T cell epitopes has to be coupled to the self-protein which will eventually disturb the B cell epitopes on the surface of the self-protein. Extensive conjugation of proteins may furthermore have the opposite effect with regard to immunogenicity (international patent application No. WO 87/00056) and the surface exposed peptide T cell epitopes may be destroyed by proteolytic enzymes during antigen processing (S. Mouritsen, Scand. J. Immunol. 30, 723, 1989), making that method less efficient than the method of the invention. By this method autoantibodies can be induced witin a few weeks (Example 2). Finally, the exact structure of such multi-conjugated self-proteins will not be chemically and pharmaceutically well-defined.

The induction of autoantibodies against TNFα by the method of the present invention has been directly compared to the autoantibody response induced when using a conjugate of TNFα and E. coli proteins, which must contain small single T cell epitope peptides as well as larger foreign carrier proteins. The autoantibody response induced by the method of the invention was induced several weeks earlier and was furthermore of a higher titer (Example 4).

Recently an improved method has been proposed for breaking the B cell autotolerance by chemical conjugation of B and optionally also peptide T cell epitopes to a high molecular weight dextran molecule (international patent application No. WO 93/23076). The disadvantages mentioned above, however, also hold true for said method, which anyway is clearly different from the method of the present invention.

Although it has been proposed previously that a well known strong T cell epitope could be inserted into a foreign protein using recombinant DNA technology (EP-A2-0 343 460) or synthetically into a peptide (WO 90/15627) in order to increase an antibody response towards that protein or peptide, it has not been proposed that this could be done with the purpose of breaking the autotolerance of the immune system. Using these methods for induction of autoantibodies one a priori would expect the same rules to be true with regard to the above-mentioned limitations of the MHC restriction of the reponse. Surprisingly, however, by using the method of the invention, it is possible to induce and equally fast and even a stronger autoantibody response against TNFα despite the fact that the inserted T cell epitope used was not restricted to the MHC molecules of the immunized mice (Example 3). The reason for this observation is not clear but may be due to the appearance of new MHC binding segments in the mutagenized area in the self-protein. However, the experiment shown in example 6 demonstrates that this is probably not the case, since synthetic peptides representing overlapping regions of the implanted ovalbumin T cell epitope in ubiquitin did not bind strongly to any of the MHC class II molecules of the H-2^k mice in which this recombinant molecule was highly immunogenic (Example 5).

Most of the potential MHC class II binding s gments of a protein are normally cryptic and will not be presented to the host T cells by the antigen presenting cells (S. Mouritsen et al, Scand. J. Immunol. 34, 421, 1991). The observed lacking correspondence between the MHC restriction of the inserted T cell epitope and the restriction of the antibody response could perhaps be due to a general disturbance of the intra-molecular competition of binding to MHC molecules by different self-protein segments. Using the herein described method non-tolerized self-protein segments may be presented to the T cells leading to breaking of the T cell as well as the B cell autotolerance towards the protein. In all the examples described below, a fragment of the self-protein was substituted with a foreign T cell epitope. This deletion followed by a substitution with an other protein fragment minimally obscure the tertiary structure of the self-proteins, but may also contribute strongly to the disturbance of said intra-molecular competition of the MHC class II binding self-segments. This concept is therefore clearly different from the above-mentioned mechanisms and methods. Independently of the operating mechanism of action by the herein described method, it is more technically advantageous compared to the known methods for breaking the B cell autotolerance, since it is possible to induce antibodies in a broad population of MHC molecules by insertion of a minimal number of different foreign T cell epitopes.

The present invention thus concerns the surprising fact that injection of recombinant proteins, which have been appropriately modulated by the insertion of one or more foreign T cell epitopes, induces a profound autoantibody response against said proteins. Surprisingly the antibody response induced is not necessarily restricted to the inserted T cell epitope. By inducing minimal tertiary structural changes in the highly conserved self-protein

ubiquitin, as well as in TNFα, foreign T cell epitopes having a length of 12-15 amino acids were inserted using genetic engineering methods. These recombinant proteins were purified, emulsified in adjuvant and injected into mice. Within only one week an autoantibody response against ubiquitin could be detected in serum from these mice. Non-modified, recombinant ubiquitin treated and injected in the same way was not able to induce a response.

By using this principle for developing vaccines against undesirable proteins, the risk of inducing allergic side-effect is reduced, and toxic self-proteins such as TNFα can simultaneously be detoxified by removing or mutating biologically active protein segments. The epitope-shielding effect described above is not a problem, and autoantibodies against ubiquitin were induced much faster as compared to the known technique, in which the self-protein is conjugated to a carrier protein or peptide. Importantly, by this method it furthermore seems possible to temporarily break the autotolerance of the T cells as well as that of the B cells of the individual, and such recombinant proteins will be self-immunogenic in a large population expressing many different MHC class II molecules.

The vaccine of the invention consists of one or more self-proteins modulated as described above and formulated with suitable adjuvants, such as calcium phosphate, saponin, quil A or biodegradable polymers. The modulated self-proteins may be prepared as fusion proteins with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

The autovaccine may i.a. be a vaccine against TNFα or γ-interferon for the treatment of patients with cachexia, e.g. cancer patients, or a vaccine against IgE for the

treatment of patients with allergy. Furthermore, it may be a vaccine against TNFα, TNFβ or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

The invention is illustrated in the following examples.

Example 1. Cloning of foreign T cell epitopes into a gene coding for ubiquitin.

An overview of this procedure is shown in fig. 1 using the T cell epitope MP7 as example. The gene sequences representing MP7 (MP7.1-C and MP7.1-NC) were synthesized as two complementary oligonucleotides designed with appropriate restriction enzyme cloning sites. The amino acid sequence of MP7 is PELFEALQKLFKHAY. The oligonucleotides were synthesized using conventional, automatic solid phase oligonucleotide synthesis and purified using agarose gel electrophoresis and low melting agarose. The desired bands were cut out from the gels, and known quantities of oligonucleotides were mixed, heated to 5°C below their theoretical melting point (usually to approximately 65°C) for 1-2 hours, and slowly cooled to 37°C. At this temperature the hybridized oligonucleotides were ligated to the vector fragments containing part of the ubiquitin gene. The subsequent analysis of positive clones using restriction fragment analysis and DNA sequencing was done by conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2 ed. CSH Laboratory Press, 1989).

Example 2. Induction of autoantibodies against ubiquitin by vaccination with modified ubiquitin molecules.

Genes containing the foreign T cell epitopes were expressed in E. coli strain, AR58 under control by the

heat sensitiv λ repressor r gulat d promotor. Expression of the recombinant ubiquitin proteins were verified using a polyclonal anti-ubiquitin antibody and Western-blotting ("Antibodies", Eds.: D. Harlow et al., CSH Laboratory Press, 1988). The recombinant proteins were purified using conventional methods (Maniatis et al., supra).

Mice were injected i.p. with 100 µg of ubiquitin or its analogs in PBS emulsified in Freunds Complete adjuvant. Booster injections of the same amount of antigen emulsified 1:1 in Freunds Incomplete adjuvant were performed i.p. at days 14 and 28. Five Balb/c mice in each group were examined and blood samples were examined for the presence of anti-ubiquitin antibodies on day 7, 14, 21, 28, 35, and 42 using conventional ELISA methodology.

The results exemplified by the antibody response against two different ubiquitin molecules containing the T cell epitopes OVA(325-336) and HEL(50-61), respectively, are shown in fig. 2. The amino acid sequence of the inserted OVA(325-336) epitope is: QAVHAAHAEINE and the amino acid sequence of the HEL(50-61) epitope is STDYGILQINSR.

A clear antibody response against native ubiquitin could be detected within only one week from the first injection of antigen reaching a maximum within 2 weeks. Anti-ubiquitin antibodies produced in rabbits by covalently conjugating ubiquitin to bovine immunoglobulin reached maximum values after a much longer immunization period (data not shown).

The antibody response against self-proteins can be increased even more by injecting self-proteins containing foreign T cell epitopes, as described in example 1, as fusion proteins with immunologically active cytokines such as e.g. granulocyte and monocyte colony stimulating factor

(GM-CSF) or interleukin 2.

Example 3. Induction of autoantibodies against tumor necrosis factors (TNF) by vaccination with appropriately modified TNF molecules.

The gene coding for the structural part of the murine TNFα protein (MR101) was obtained by Polymerase Chain Reaction (PCR) cloning of the DNA. In the MR103 TNFα mutant the ovalbumin (OVA) H-2^d restricted T cell epitope sequence 325-334 (QAVHAAHAET) replaces the amino acids 26-35 in the cloned TNFα sequence, a substitution of an amphiphatic α-helix. Substitutions in this region of the TNFα detoxifies the recombinant protein (X. Van Ostade et al., Nature 361, 266-269, 1993). In the MR105 TNFα mutant the H-2^k restricted T cell epitope from hen eggwhite lysozyme (HEL), amino acid sequence 81-96 (SALLSSDITASVNCAK) replaces the amino acids 5-20 in the cloned TNFα sequence. In the MR106 TNFα mutant the same epitope, amino acid sequence 81-95 (SALLSSDITASVNCA) replaces the amino acids 126-140 in the cloned TNFα sequence. The genetic constructions are described in Fig. 3. Different techniques compared to the technique described in example 1 were used for exchanging parts of the TNFα gene with DNA coding for T cell epitopes. The MR105 and 106 constructs were made by introducing the mutant sequence by PCR recloning a part of the TNFα gene flanking the intended site for introducing the T cell epitope. The mutant oligonucleotide primer contained both a DNA sequence homologous to the TNFα DNA sequence as well as a DNA sequence encoding the T cell epitope. The PCR recloned part of the TNFα gene was subsequently cut with appropriate restriction enzymes and cloned into the "wild type" MR101 gene. the MR103 construction was made by a modification of the "splicing by overlap extension" PCR technique (R. M. Horton et al., Gene 77, 61, 1989). Here

two PCR products are produced, each covering a part of the TNFα gene, and additionally each PCR product contains half of the T cell epitope sequence. The complete mutant TNFα gene was subsequently made by combining the two PCR products in a second PCR. Finally, the complete genetic constructions were inserted into protein expression vectors. Subsequently, all genetic constructions were analyzed by restriction fragment analysis and DNA sequencing using conventional methods ("Molecular Cloning", Eds,: T. Maniatis et al. 2.ed. CSH Laboratory Press, 1989). The recombinant proteins were expressed in E.coli and purified by conventional protein purification methods.

Groups of BALB/c (MHC haplotype, H-2^d) and C3H (MHC haplotype, H-2^k) mice, respectively, were immunized subcutaneously with 100 μg of semi-purified MR103 and MR106 emulsified in Freunds' complete adjuvant. Every second week the immunizations were repeated using incomplete Freunds' adjuvant. All mice developed an early and strong antibody response against biologically active MR101. This was measured by a direct ELISA method using passively adsorbed 100% pure MR101 (Fig. 4). Control mice immunized with MR101 and PBS, respectively, showed no antibody reactivity towards MR101.

Strinkingly, the response was not MHC restricted corresponding to the implanted T cell epitopes, since both mice strains responded well to MR103 and MR106 (Fig. 4). Taken together these results illustrate (a) the ability of the method of the invention to induce autoantibodies towards a secreted autoprotein and (b) the improved efficiency of the herein described method with regard to inducing a response in a broader MHC population than predicted by the MHC binding ability of the inserted T cell epitopes. The immune response against the recombinant proteins MR103 and MR106 was much stronger and more high-titered compared to

aldehyde conjugated MR101 (see Example 4).

Example 4. Induction of autoantibodies against TNFα by the method of the invention compared to conjugation to E. coli proteins.

Semi-purified recombinant murine TNFα (MR101) was conjugated to E. coli proteins in PBS, pH 7.4, using 0.5% formaldehyde. Conjugation of the proteins was confirmed by SDS-PAGE. These conjugates were subsequently used for immunization of C3H mice. Another group of C3H mice was vaccinated only with semi-purified non-conjugated MR105, and about 100 μg of recombinant TNFα were emulsified 1:1 in Freunds' complete adjuvant and injected subcutaneously in each mouse. MR105 is biologically inactive as judged by the L929 bioassay for TNFα. In subsequent immunizations every second week incomplete Freunds' adjuvant was used. Both groups eventually developed autoantibodies against highly purified biologically active MR101 as determined by ELISA, but the immune response against non-conjugated MR105 was induced earlier and was of a higher titer (Fig. 5).

Example 5. The possible MHC class II binding of peptides representing overlapping sequences of self-protein as well as of the ovalbumin T cell epitope inserted in ubiquitin.

Peptide-MHC complexes were obtained by incubaing ^{125}I-labelled peptide (10-100 nM) with affinity purified MHC class II molecules (2-10 μM) at room temperature for 3 days (S. Mouritsen, J. Immunol. 148, 1438-1444, 1992). The following peptides were used as radiolabelled markers of binding: Hb(64-76)Y which binds strongly to the E^k molecule and HEL(46-61)Y which binds strongly to th A^k

molecule. These complexes were co-incubated with large amounts of cold peptide (> 550 µm) which is sufficient to inhibit totally all immunologically relevant MHC class II binding. Either the same peptides were used, or three different overlapping peptides were used, said peptides representing the flanking regions as well as the entire OVA(325-336) T cell epitope which was substituted into ubiquitin (see Example 2). The three peptides were: TITLEVEPSQAVHAA (U(12-26)), PSQAVHAAHAEINEKE (U(19-34)) and HAEINEKEGIPPDQQ (U(27-41)). The reaction buffer contained 8 mM citrate, 17 mM phosphate, and 0.05% NP-40 (pH 5) and peptide-MHC class II complexes were separated (in duplicate) from free peptide by gel filtration using G25 spun columns. Both the radioactivities of the excluded "void" volume and of the included volume were measured by gamme spectrometry. The competitive inhibition of maximal binding (in percent) by addition of cold peptide was calculated. The results are shown in Table I.

Table I.

Peptid/ MHC	Hb(64-76)	HEL(46-61)	U(12-26)	U(19-34)	U(27-41
A^k	28.6	<u>97.4</u>	35.3	44.6	7.8
E^k	<u>92.6</u>	0.0	45.6	12.2	0.0

It can be seen that total inhibition of the binding of the radiolabelled peptides Hb(64-76)Y and HEL(46-61)Y to E^k and A^k respectively could only be achieved using cold versions of the same peptides. Although some inhibition of binding was seen by U(12-26) and U(19-434) using these extreme amounts of cold peptide, it is likely that the

affinity of these peptides to the $H-2^k$ MHC class molecules is very low. Therefore this seems not to be sufficient to explain the strong immunogenicity in the $H-2^k$ mouse strain of the ubiquitin analog containing the ovalbumin T cell epitope. More likely, other and previously non-tolerized self-epitopes are presented to the T cell in these animals.

Example 6. Treatment of diabetes of inflammatory disease by vaccination with appropriately modified TNFα molecules.

Genes coding for TNFα are modified by insertion of appropriate gene segments coding for T cell epitopes derived from e.g. tetanus toxin or influenza hemagglutinin. Such genes are expressed in appropriate expression vectors in e.g. E. coli or insect cells. The recombinant TNFα proteins were purified using conventional methods ("Molecular Cloning", Eds.: T. Maniatis et al. 2. ed. CSH Laboratory Press, 1989).

Optionally such recombinant proteins can be coupled to immunologically active cytokines such a GMCSF or interleukin 2.

The recombinant proteins can be formulated with appropriate adjuvants and administered as an anti-TNFα vaccine to patients suffering from diseases where TNFα is important for the pathogenesis. The induced anti-TNFα antibodies will thereby affect the diseases.

One example of said diseases is the chronic inflammatory diseases such as e.g. rheumatoid arthritis where TNFα is believed to play an important role (reviewed in: F.M. Brennan et al., Br. J. Rheumatol. 31, 293-298, 1992). TNFα is also believed to play an important role in the cachec-

tic conditions seen in cancer and in chronic infectious diseases such as AIDS (reviewed in M. Odeh. J. Intern. Med. 228, 549-556, 1990). It is also known that TNF participates in septic shock (reviewed in: B.P. Giroir, Crit. Care. Med., 21, 780-789, 1993). Furthermore, it has been shown that TNFα may paly a pathogenetic role in the development of type II diabetes mellitus (CH Lang et al., Endocrinology 130, 43-52, 1992).

Legends to figures

Fig. 1. Schematic overview of the cloning strategy used in the construction of a ubiquitin gene with an implanted foreign T cell epitope (MP7). Restriction enzyme digestions, hybridization and ligation procedures are indicated with arrows. Fragment sizes are shown in parentheses.

Fig. 2. Reactivity toward immobilized bovine ubiquitin in sera from mice immunized with recombinant ubiquitin and analogs containing the implanted T cell epitopes OVA(323-339) and HEL (50-61), respectively. Fig. 2a) sera from Balb/c mice immunized with recombinant ubiquitin containing OVA(325-336). Fig. 2b) sera from Balb/c mice immunized with recombinant ubiquitin containing the T cell epitope HEL(50-61). Fig. 2c) sera from Balb/c mice immunized with recombinant non-modified ubiquitin. Sera (diluted 1:100) were tested in a standard ELISA assay using non-modified bovine ubiquitin immobilized on the solid phase.

Fig. 3. Schematic overview of the cloning strategy used in the construction of the recombinant TNFα mutants. PCR products and restriction enzyme digestions are indicated.

Fig. 4. Induction of TNFα autoantibodies by vaccination of Balb/c or C3H mice with semipurified MR103 and MR106. The antibody titers were measured by ELISA and expressed as arbitrary units (AU) referring to a strong standard anti-serum from one mouse. The plotted values represent a mean titer for 5 animals. Freunds complete adjuvant was used as adjuvant for the first immunization. All subsequent immunizations at 14 days internvals were done with Freunds incomplete adjuvant. Mice immunized in parrallel with native MR101 in PBS did not develop detectable TNFα auto-antibodies (data not shown). Non-detectable antibody titers were assigned the titer value 1.

Fig. 5. Anti TNFα autoantibodies induced by vaccination with non-conjugated MR105 and MR101 conjugated to E. coli proteins, respectively. C3H mice and Balb/c mice were immunized with both preparations. The immunizations, measurements and calculations of mean antibody titers were done as described in example 4.

C l a i m s :

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1. A method for the modulation of self-proteins by inducing antibody responses against such proteins, c h a r a c t e r i z e d in that one or more foreign T cell epitopes are inserted in such proteins by molecular biological means, thereby rendering said proteins immunogenic.

2. A method according to claim 1, c h a r a c t e r - i z e d in that immunodominant T cell epitopes from tetanus toxoid or diphtheria toxoid are inserted in said proteins.

3. An autovaccine against undesirable proteins in humans or animals, c h a r a c t e r i z e d in that it consists of one or more self-proteins modulated according to claim 1 or 2 and formulated with pharmaceutically acceptable adjuvants, such as calcium phosphate, saponin, quil A and biodegradable polymers.

4. An autovaccine according to claim 3, c h a r a c - t e r i z e d in that the modulated self-proteins are prepared as fusion proteins with suitable, immunologically active cytokines, such as GM-CSF or interleukin 2.

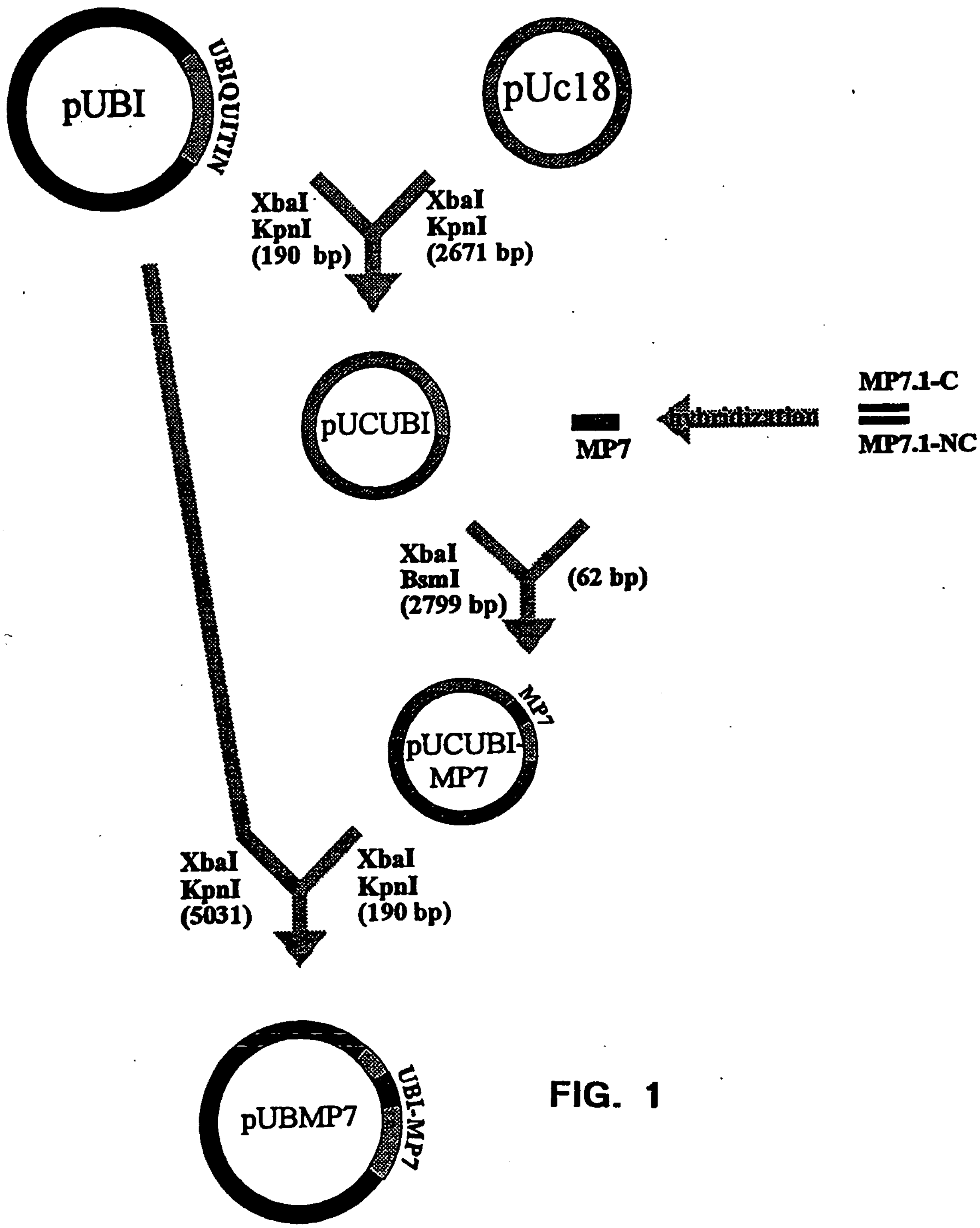
5. An autovaccine according to claim 3, c h a r a c - t e r i z e d in that it is a vaccine against TNFα or γ-interferon for the treatment of patients with cachexia, e.g. cancer patients.

6. An autovaccine according to claim 3, c h a r a c - t e r i z e d in that it is a vaccine against IgE for the treatment of patients with allergy.

7. An autovaccine according to claim 3, c h a r a c t e r i z e d in that it is a vaccine against TNFα, TNFβ or interleukin 1 for the treatment of patients with chronic inflammatory diseases.

8. An autovaccine according to claim 7, c h a r a c t e r i z e d in that it is a vaccine for treatment of patients with rheumatoid arthritis or inflammatory bowel disease.

9. An autovaccine according to claim 3 or 4, c h a r a c t e r i z e d in that it is a vaccine against TNFα for the treatment of diabetes mellitus.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UBIQUITIN
pUc18
XbaI
KpnI
(190 bp)
XbaI
KpnI
(2671 bp)
pUCUBI
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hybridization
MP7.1-C
MP7.1-NC
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FIG. 1

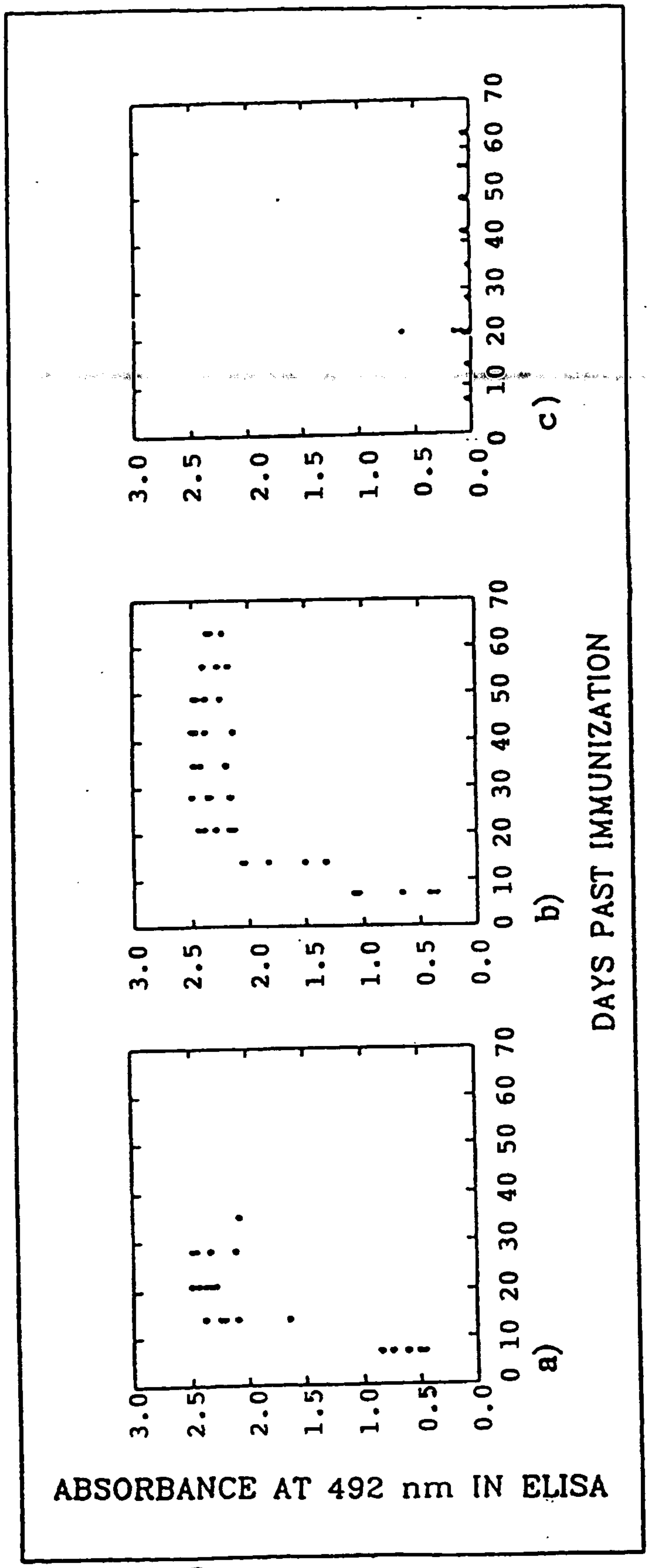
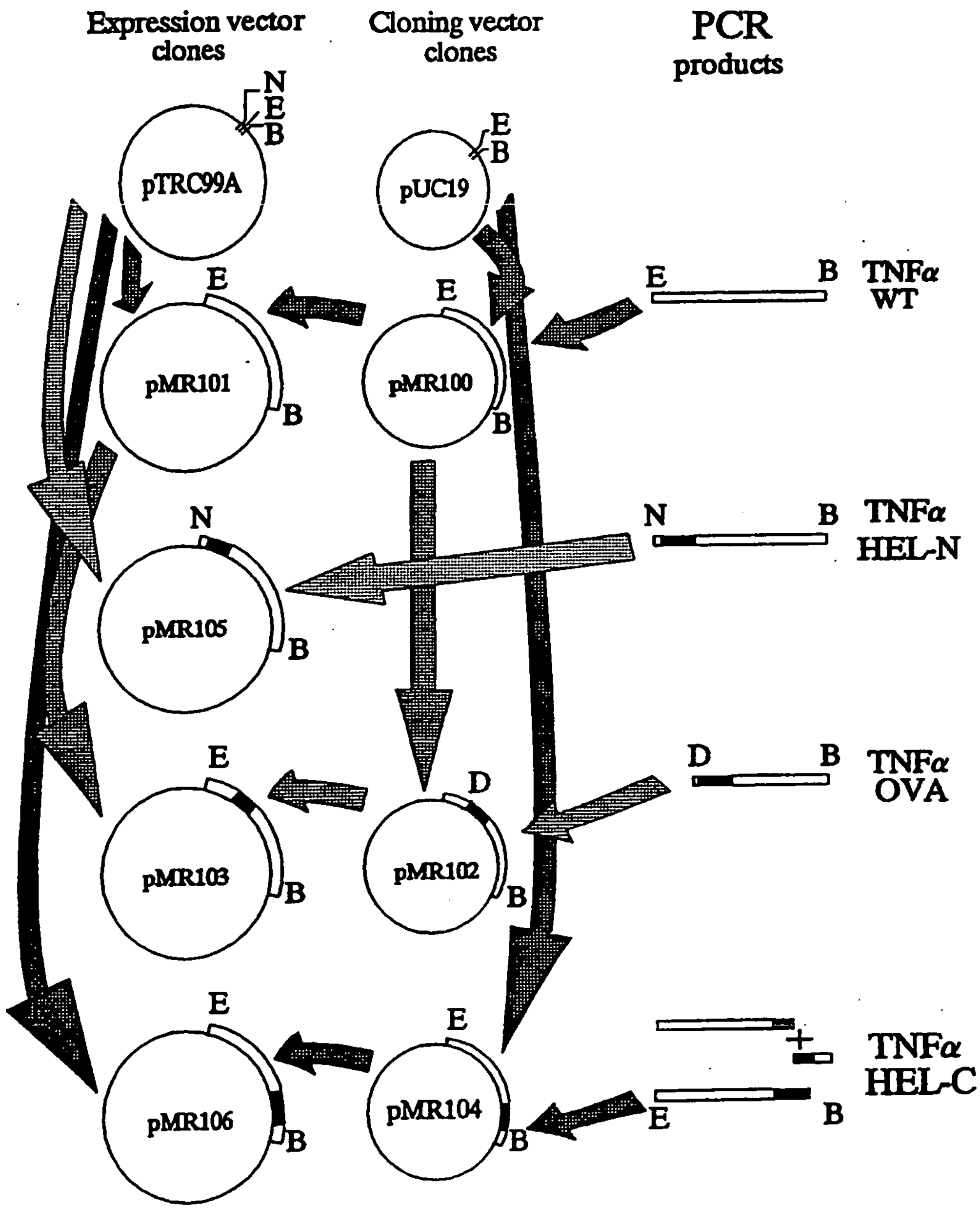

ABSORBANCE AT 492 nm IN ELISA
DAYS PAST IMMUNIZATION
a)
b)
c)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0 10 20 30 40 50 60 70

FIG. 2

Cloning strategy for murine TNFα mutants.



Restriction enzyme symbols: E: EcoRI, B:BamHI, N: NcoI, D: DraIII.

FIG. 3

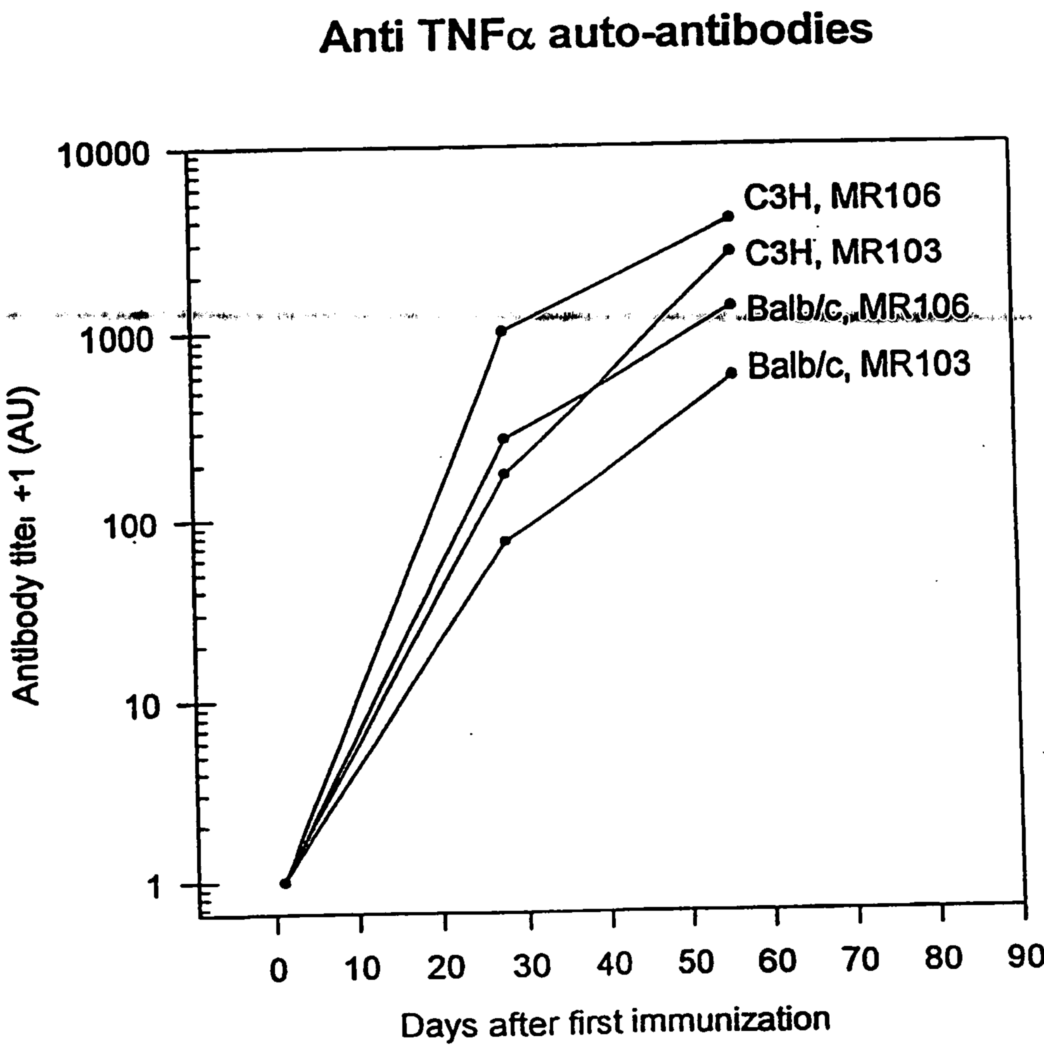

Anti TNFα auto-antibodies
10000
1000
100
10
1
Antibody titer +1 (AU)
C3H, MR106
C3H, MR103
Balb/c, MR106
Balb/c, MR103
0
10
20
30
40
50
60
70
80
90
Days after first immunization

FIG. 4

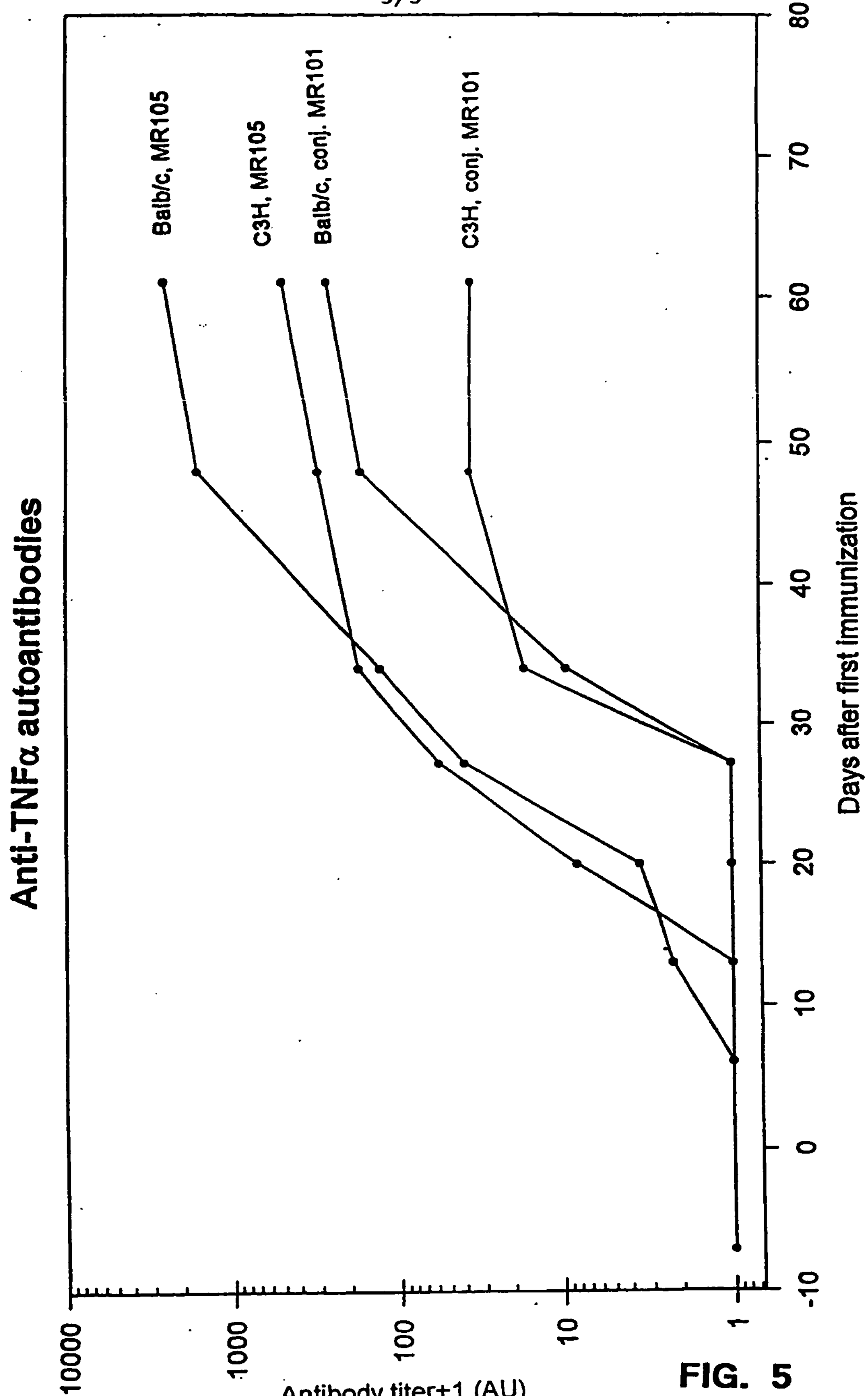

Anti-TNFα autoantibodies
Balb/c, MR105
C3H, MR105
Balb/c, conj. MR101
C3H, conj. MR101
Antibody titer+1 (AU)
10000
1000
100
10
1
Days after first immunization
-10
0
10
20
30
40
50
60
70
80

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00318

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/00, A61K 39/385, C07K 19/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, US FULLPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	National Library of Medicine database, Medline, File Med 93, NLM Accession no. 93013892, Sad S. et al: "Bypass of carrier-induced epitope--specific suppression using a T-helper epitope", & Immunology 1992 Aug; 76(4): 599-603	1-3
Y		1-9
	--	
X	WO, A1, 9219746 (CSL LIMITED), 12 November 1992 (12.11.92), page 3, line 3 - line 14, see claims	1-3
Y		1-9
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" ertier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 January 1995	01 -02- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl Olof Gustafsson Telephone No. +46 8 782 25 00

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International application No.
PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0343460 (F. HOFFMANN-LA ROCHE & CO. AG.), 29 November 1989 (29.11.89), page 2, line 31 - page 3, line 49; page 5, line 6 - line 13	1-3
Y		1-3
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X	WO, A1, 9015627 (BOARD OF REGENTS, THE UNIVERSITY OF TEXASSYSTEM), 27 December 1990 (27.12.90), page 9, line 24 - line 28	1-3
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A	Dialog Information Services, file 154, Medline, accession no. 08270666, Medline accession no. 92408666, Lowenadler B. et al: "T and B cell responses to chimeric proteins containing heterologous T helper epitopes inserted at different positions", & Mol Immunol Oct 1992, 29 (10) p1185-90	1
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A	Methods in Enzymology, Volume 178, 1989, M. J. Francis et al, "Peptide Vaccines Based on Enhanced Immunogenicity of Peptide Epitopes Presented with T-Cell Determinants or Hepatitis B Core Protein" page 659 - page 676	1-3
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A	SCIENCE, Volume 249, July 1990, H. M. Etlinger et al, "Use of Prior Vaccinations for the Development of New Vaccines" page 423 - page 425	1-3
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A	Dialog Information Services, file 154, Medline, Dialog accession no. 07947371, Medline accession no. 92085371, Schodel F. et al: "The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity published erratum appears in J Virol 1992 Jun; 66(6):3977, & J Virol (UNITED STATES) Jan 1992, 66 (1) p106-14	1-3
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Dialog Information Services, file 154, Medline, Dialog accession no. 07446388, Medline accession no. 90353388, Lowenadler B. et al: "Enhanced immunogenicity of recombinant peptide fusions containing multiple copies of a heterologous T helper epitope", & Eur J Immunol Jul 1990, 20 (7) p1541-5 --	1-3
P,X	WO, A1, 9323076 (THE JOHNS-HOPKINS UNIVERSITY), 25 November 1993 (25.11.93), see claims 1, 6-15 and pages 7-9 --	1-3
X	US, A, 9218150 (ANERGEN, INC.), 29 October 1992 (29.10.92), page 7 - page 8; page 16, line 35 - page 20 --	1-3
X	WO, A1, 9305810 (HELLMAN, LARS, T.), 1 April 1993 (01.04.93), page 5, line 29 - line 33 --	1-3,6
X	WO, A1, 8912458 (CELL MED, INC.), 28 December 1989 (28.12.89), see pages 1-3, page 8, last two lines, page 12, page 28, third paragraph and claims 1,4,9, 22 and 29	1,3,4,7-9
Y	 --	1-9
Y	National Library of Medicine (NLM), file Medline, Medline accession no. 88131024, Murphy JR et al: "Interleukin 2 toxin: a step toward selective immunomodulation", Am J Kidney Dis 1988 Feb;11(2):159-62 --	4
A	WO, A1, 8303971 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE), 24 November 1983 (24.11.83), see claims 8-10 --	4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0269455 (TAKEDA CHEMICAL INDUSTRIES, LTD.), 1 June 1988 (01.06.88), see page 5, lines 6-28 and claims --	6
Y	US, A, 4684623 (JAMES W. LARRICK ET AL), 4 August 1987 (04.08.87) --	5,7-9
Y	WO, A2, 8807869 (STICHTING REGA VZW), 20 October 1988 (20.10.88) --	5
A	WO, A1, 9102005 (TURANO, ADOLFO), 21 February 1991 (21.02.91) --	5
Y	WO, A1, 9010707 (JONKER, MARGREET), 20 Sept 1990 (20.09.90) --	4,5,7-9
Y	WO, A1, 9101330 (SCHERING CORPORATION), 7 February 1991 (07.02.91) --	4
A	US, A, 4772685 (JOHN A. SCHMIDT ET AL), 20 Sept 1988 (20.09.88) -- --------	1,3,4

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International application No.
PCT/DK 94/00318

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☒ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

1) Claims 1-3
A method for the modulation of self proteins by insertion of a T-cell epitope into the protein and an autovaccine.

2) Claims 4,5,7 and 8
An autovaccine comprising a fusion protein of a foreign T-cell epitope and a self protein prepared as fusion proteins with suitable, immunologically active cytokines.

3) Claim 6
Autovaccine comprising a foreign T-cell epitope inserted in IgE

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International application No.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A1- 9323076	25/11/93	NONE	
US-A- 9218150	29/10/92	NONE	
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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International application No.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU-A- 6148790	22/02/91
		CA-A- 2062975	15/01/91
		EP-A- 0409091	23/01/91
		EP-A- 0482086	29/04/92
		JP-T- 4503815	09/07/92
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		JP-A- 62096498	02/05/87
		US-A- 4994553	19/02/91